Stathmin 1 Induces Murine Hepatocyte Proliferation and Increased Liver Mass

Enpeng Zhao,[1] Yang Shen,[2] Muhammad Amir,[2] Alton B. Farris,[3] and Mark J. Czaja [2]

The endogenous cellular signals that initiate the transition of hepatocytes from quiescence to proliferation remain unclear. The protein stathmin 1 (STMN1) is highly expressed in dividing cells, including hepatocytes, and functions to promote cell mitosis through physical interactions with tubulin and microtubules that regulate mitotic spindle formation. The recent finding that STMN1 mediates the resistance of cultured hepatocytes to oxidant stress led to an examination of the expression and function of this protein in the liver *in vivo*. STMN1 messenger RNA (mRNA) and protein were essentially undetectable in normal mouse liver but increased markedly in response to oxidant injury from carbon tetrachloride. Similarly, levels of STMN1 mRNA and protein were increased in human livers from patients with acute fulminant hepatic failure. To determine STMN1 function in the liver *in vivo*, mice were infected with a control or *Stmn1*-expressing adenovirus. *Stmn1* expression induced spontaneous liver enlargement with a doubling of the liver to body weight ratio. The increase in liver mass resulted, in part, from hepatocellular hypertrophy but mainly from an induction of hepatocyte proliferation. STMN1 expression led to marked increases in the numbers of 5-bromo-2′-deoxyuridine-positive and mitotic hepatocytes and hepatic nuclear levels of cyclins and cyclin-dependent kinases. STMN1-induced hepatocyte proliferation was followed by an apoptotic response and a return of the liver to its normal mass. *Conclusion:* STMN1 promotes entry of quiescent hepatocytes into the cell cycle. STMN1 expression by itself in the absence of any reduction in liver mass is sufficient to stimulate a hepatic proliferative response that significantly increases liver mass. (*Hepatology Communications* 2020;4:38-49).

Mammalian liver size is precisely controlled and maintained in proportion to total body mass by mechanisms that are poorly understood. In the normal liver, hepatocytes exist in a nonproliferative state with virtually all the cells in the nonreplicative gap $(G)_0$ phase of the cell cycle. These quiescent cells enter the cell cycle only in response to signals generated by a significant loss of liver mass resulting from either hepatocyte injury and death or surgical removal. No single factor has been demonstrated to be capable of triggering liver growth in response to a loss of hepatic mass. Rather, hepatic proliferation is thought to involve the combined effects of a number of cytokines and growth factors.[(1)] Early inflammatory cytokine signals may function to prime hepatocytes to enter into the G_1 phase of the cell cycle. After this priming event, hepatocytes become competent to respond to growth factors that trigger progression through G_1 and the remainder of the cell cycle. Once normal liver mass is restored, the hepatocytes exit the cell cycle and resume their quiescent state. Hepatic proliferation has been thought, therefore, to occur only in the setting of a reduction in liver mass that initiates this complex interaction of multiple factors and signaling pathways that trigger hepatocyte entry, progression, and exit from the cell cycle.

Stathmin 1 (STMN1) is a ubiquitous cytoplasmic protein that regulates mitosis through its physical

Abbreviations: AdGFP, adenovirus expressing green fluorescent protein; AdStath, adenovirus expressing mouse stathmin 1; ALT, alanine aminotransferase; BrdU, 5-bromo-2′-deoxyuridine; CDK, cyclin-dependent kinase; mRNA, messenger RNA; PCR, polymerase chain reaction; phospho, phosphorylated; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; Ser, serine; STMN1, stathmin 1.

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modulation of microtubules.[2,3] STMN1 effects on microtubule equilibrium regulate microtubule polymerization required for formation of the mitotic spindle that allows chromosome segregation and cell division. Dephosphorylated STMN1 destabilizes microtubules by sequestering free tubulin or promoting microtubule catastrophe, whereas phosphorylated STMN1 enhances microtubule polymerization.[4,5] STMN1 can therefore either block or promote spindle formation depending on the levels of both total STMN1 expression and its phosphorylation. In addition to involvement in mitotic spindle formation, STMN1 has been implicated in the regulation of cell proliferation through effects on cell-cycle factors.[6] The significance of these findings to the function of STMN1 in normal cell proliferation *in vivo* is unclear as these investigations have been performed exclusively in cultured transformed cells. The *Stmn1* global knockout mouse is uninformative regarding the normal physiologic function of this protein because the knockout develops normally,[7] likely due to compensatory effects of other stathmin family members.[6]

Indicative of a function in cell proliferation is that STMN1 is highly expressed in rapidly proliferating cells.[8] Consistent with the quiescent state of adult liver and a role for STMN1 in hepatocyte proliferation are the findings that hepatic *Stmn1* expression is high during embryologic liver growth, absent after birth, and reoccurs in hepatocytes in response to the regenerative stimulus of surgical partial hepatectomy.[9-11] In addition, *Stmn1* is expressed in the majority of hepatocellular carcinomas and correlates with poor prognostic features, such as high tumor grade, vascular invasion, and early recurrence.[12,13] Despite this suggestive evidence of STMN1 involvement in hepatocyte proliferation, whether STMN1 functions *in vivo* to promote the proliferation of normal hepatocytes or any other nontransformed cell type is unknown.

Recent studies from our laboratory demonstrated that STMN1 expression is induced in cultured hepatocytes in response to oxidative stress and protects against oxidant injury.[14] In light of this finding, we examined the involvement of STMN1 in liver injury *in vivo*. STMN1 expression is induced in response to murine and human liver injury. Adenoviral expression of STMN1 in hepatocytes *in vivo* triggers hepatocyte hypertrophy and spontaneous entry into the cell cycle, causing a significant increase in liver size. These findings demonstrate a unique ability of STMN1 to drive hepatocytes into the cell cycle and promote an increase in liver mass in the absence of any reduction in hepatic size.

Materials and Methods

ANIMALS

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained under 12-hour light/dark cycles with unlimited access to food (PicoLab Rodent Diet 20 #5053; Lab Supply, Fort Worth, TX) and water. Male mice aged 12-14 weeks were treated with 1 mL/kg CCl_4 diluted 1:10 in mineral oil (MilliporeSigma, St. Louis, MO).[15] All animal studies were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine or the Emory University School of Medicine and followed the National Institutes of Health (NIH) guidelines for animal care.

HUMAN LIVERS

Human liver samples were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN), which is funded by NIH Contract HSN276201200017C, as described.[16] Normal livers

ARTICLE INFORMATION:

From the [1]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY; [2]Division of Digestive Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, GA; [3]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Mark J. Czaja, M.D.
Emory University School of Medicine
615 Michael Street, Suite 201
Atlanta, GA 30322
E-mail: mark.j.czaja@emory.edu
Tel.: +1-404-712-2867

were donor livers unused for reasons other than the presence of liver abnormalities. Diseased liver specimens were explants from patients undergoing transplantation for acute fulminant hepatic failure. The studies were approved by the Albert Einstein College of Medicine Committee on Clinical Investigation.

QUANTITATIVE REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total mouse or human liver RNA was isolated with the commercial kit RNeasy Plus (QIAGEN, Valencia, CA). Reverse transcription with 1 μg RNA was carried out in an Eppendorf Mastercycler (Hamburg, Germany), using a high-capacity complementary DNA reverse-transcription kit (Thermo Fisher Scientific, Waltham, MA). Annealing of primers was done at 25˚C for 10 minutes, followed by elongation at 37˚C for 2 hours and inactivation of the enzyme at 85˚C for 5 minutes. Negative controls with no added transcriptase were performed in parallel. Polymerase chain reaction (PCR) was performed in a 7500 Fast Real-Time PCR System (ABI, Foster City, CA). Primers were purchased from Integrated DNA Technologies (Coralville, IA) with the sequences shown in Table 1. PCR was carried out using Power SYBR Green Master Mix (Thermo Fisher Scientific). Taq polymerase was activated at 95˚C for 10 minutes. The cycling parameters were denaturation at 95˚C for 30 seconds and annealing and extension at 60˚C for 1 minute (for 40 cycles). Data were analyzed using the $2^{-\Delta\Delta CT}$ method for relative quantification. STMN1 messenger RNA (mRNA) levels were normalized to those for glyceraldehyde-3-phosphate dehydrogenase.

PROTEIN ISOLATION AND WESTERN BLOTTING

Total protein was isolated from mouse and human livers, as described.[17,18] Membranes were probed with antibodies to β-actin (#A5441; MilliporeSigma), caspase 3 (#9665; Cell Signaling, Beverly, MA), caspase 7 (#9492; Cell Signaling), cyclin A (#sc-596; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin B1 (#sc-595; Santa Cruz Biotechnology), cyclin D1 (#MA1-24750; Thermo Fisher Scientific), cyclin D2 (#sc-593; Santa Cruz Biotechnology), cyclin D3 (#sc-182; Santa Cruz Biotechnology), cyclin-dependent kinase (CDK) 2 (#sc-163; Santa Cruz Biotechnology), CDK4 (#sc-260; Santa Cruz Biotechnology), E2F2 (#sc-633; Santa Cruz Biotechnology), Fos-related antigen 1 (#sc-183; Santa Cruz Biotechnology), total STMN1 (#3352; Cell Signaling), phosphorylated (phospho)-Serine (Ser)-16 STMN1 (#3353; Cell Signaling), phospho-Ser-38 STMN1 (#3426; Cell Signaling), and tubulin (#9411; Cell Signaling).

TABLE 1. PCR PRIMER SEQUENCES

E1a	Forward	5′-CTGATCGAAGAGGTACTGGCT-3′
	Reverse	5′-CTCCTCACCCTCTTCATCCTC-3′
GAPDH (human)	Forward	5′-GGTGTGAACCATGAGAAGTATGA-3′
	Reverse	5′-GAGTCCTTCCACGATACCAAAG-3′
Gapdh (mouse)	Forward	5′-AGGTCGGTGTGAACGGATTTG-3′
	Reverse	5′-TGTAGACCATGTAGTTGAGGTCA-3′
STMN1 (human)	Forward	5′-GCCTGTCGCTTGTCTTCTATT-3′
	Reverse	5′-GTGCCTCTCGGTTCTCTTTATT-3′
Stmn1 (mouse)	Forward	5′-GCCAGTCTCTTGTCTTCTGTT-3′
	Reverse	5′-CCTCCCGGTTCTCTTTGTTAG-3′

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ADENOVIRUSES

A control adenovirus that expresses green fluorescent protein (AdGFP; #1060) and an adenovirus that expresses mouse STMN1 (AdStath; #273354) were commercially obtained (Vector Biolabs, Malvern, PA). Adenoviruses were amplified in HEK293 cells (American Type Culture Collection, Manassas, VA), isolated, and titered by plaque assay, as described.[19] Mice received 2.5×10^8 plaque-forming units/mouse of adenovirus intravenously by tail vein. To detect E1A-positive wild-type adenovirus contamination, plaque assays were performed in A549 cells (American Type Culture Collection).

LIVER HISTOLOGY, IMMUNOFLUORESCENCE, AND 5-BROMO-2′-DEOXYURIDINE STAINING

Liver samples were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylin and eosin for histologic evaluation by a single pathologist in a blinded fashion. Numbers of mitotic and apoptotic hepatocytes were determined by manual

count. The degree of inflammation was graded semiquantitatively on a sliding scale as follows: 0, absent; 0.5, minimal; 1, mild; 1.5, mild to moderate; 2, moderate; 2.5, moderate to marked; and 3, marked.

For STMN1 and E-cadherin immunofluorescence, 10 μm paraffin sections were deparaffinized in xylene and rehydrated through graded ethanol washes. Samples were boiled in 10 mM citric acid for 30 minutes followed by a 60-minute cool down, blocked for 2 hours, and incubated with anti-STMN1 or anti-E-cadherin (#3195; Cell Signaling) antibody overnight. After washing, sections were incubated with cyanine-3-conjugated secondary antibody (#111-165-144; Jackson ImmunoResearch, West Grove, PA) for 1 hour and visualized under fluorescence microscopy. Hepatocyte area was quantitated using ImageJ.

For 5-bromo-2′-deoxyuridine (BrdU) staining, deparaffinized sections were prepared in the same manner from mice injected intraperitoneally with BrdU (100 mg/kg; MilliporeSigma) 2 hours before being killed. The samples were incubated with an anti-BrdU antibody (#NA61; MilliporeSigma) and 3,3′-diaminobenzidine (US Biological, Salem, MA).

ALANINE AMINOTRANSFERASE ASSAY

Serum alanine aminotransferase (ALT) levels were measured using a commercial kit (TECO Diagnostics, Anaheim, CA).

STATISTICAL ANALYSIS

All numerical results are reported as means ± SE and derived from at least three independent experiments. Groups were compared by the unpaired Student *t* test, with statistical significance defined as $P < 0.05$ using Sigma Plot (Jandel Scientific, San Rafael, CA).

Results

HEPATIC STMN1 EXPRESSION IS INDUCED DURING MURINE AND HUMAN LIVER INJURY

Oxidative stress is a central mechanism of hepatic injury in a number of forms of liver disease.[20] Recently, we demonstrated that STMN1 is a critical mediator of hepatocyte resistance to death from oxidative stress *in vitro*.[14] These findings led us to examine whether STMN1 expression is regulated *in vivo* during mouse and human liver injury. In the mouse model of acute oxidant injury induced by the hepatotoxin CCl_4, levels of *Stmn1* mRNA, as determined by quantitative real-time reverse-transcription (qRT)-PCR, increased significantly within 24 hours and continued to rise throughout the postinjury recovery period to 72 hours (Fig. 1A). No STMN1 protein was seen on immunoblots of normal mouse liver, but protein was detectable at 48 hours and further increased at 60-72 hours after CCl_4 administration (Fig. 1B). Levels of Ser-16- and Ser-38-phospho-STMN1 increased in parallel with total protein content, indicating that liver injury also induced posttranslational changes in STMN1 (Fig. 1B). Findings that increased mRNA and protein expression occur after the 36-48-hour peak of liver injury suggest that STMN1 functions during the reparative phase after liver injury in this model.

Levels of STMN1 were also examined in acute human liver injury by comparing mRNA and protein expression in normal liver to that in livers of patients with acute fulminant hepatic failure. *STMN1* mRNA levels were increased 30-fold in injured human livers compared to normal livers (Fig. 1C). Identical to the mouse findings, STMN1 protein was not expressed in normal human liver but total and Ser-16- and Ser-38-phospho-STMN1 were highly expressed in the injured livers (Fig. 1D). This increase in STMN1 expression was not a nonspecific stress response to liver injury as other proteins, such as Fos-related antigen 1, were reduced in livers from patients with hepatic failure (Fig. 1D). Similar findings in mouse and human liver demonstrate that a marked induction of total and phospho-STMN1 expression occurs during hepatic injury, suggesting a potential physiologic role for this protein in the liver's response to damage.

STMN1 TRIGGERS A SPONTANEOUS INCREASE IN LIVER MASS

The finding of STMN1 expression in the CCl_4 model after the peak of liver injury and during the period of liver repair together with the potential of STMN1 to regulate cell proliferation suggested that

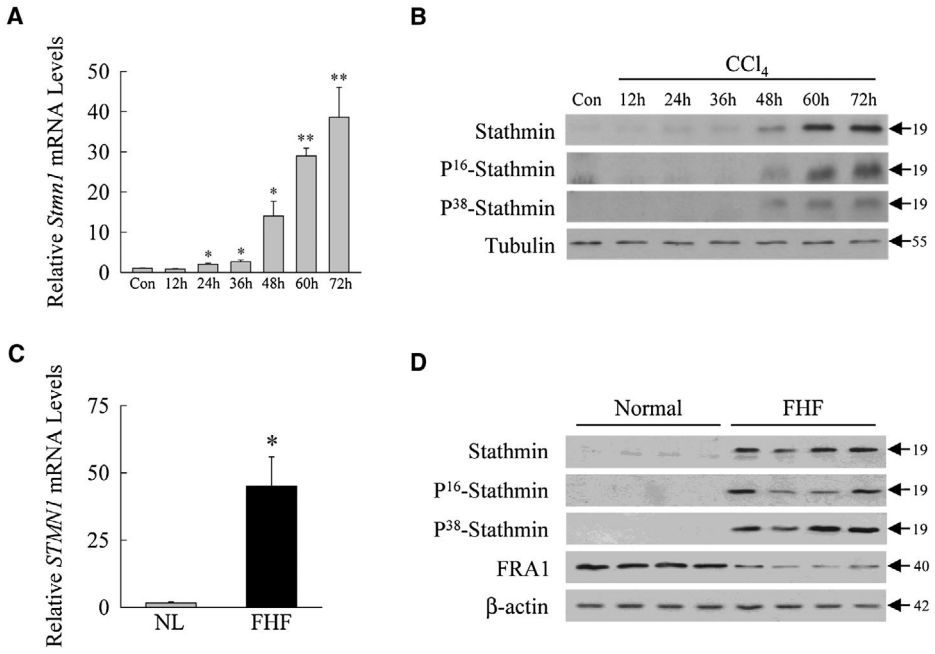

FIG. 1. Hepatic STMN1 mRNA and protein expression are increased in mouse and human liver injury. (A) Hepatic *Stmn1* mRNA levels relative to uninjected control mice in mice treated for the indicated number of hours with CCl_4 by qRT-PCR (**P* < 0.01, ***P* < 0.0002, compared to control; n = 3-6). (B) Immunoblots of total hepatic protein from an untreated control mouse and CCl_4-treated mice probed for total STMN1, Ser-16- and Ser-38-phospho-STMN1, and the loading control tubulin. (C) Relative levels of *STMN1* mRNA in normal human livers and livers from patients with fulminant hepatic failure (**P* < 0.007, compared to normal; n = 4). (D) Total human liver protein from the same livers immunoblotted for total and phosphorylated STMN1, Fos-related antigen 1, and β-actin as a loading control. Arrows in (B) and (D) indicate the respective protein bands and molecular weights in kilodaltons. Abbreviations: Con, control; FHF, fulminant hepatic failure; FRA1, Fos-related antigen 1; h, hours; NL, normal liver; P^{16}-stathmin, serine-16 phosphorylated stathmin 1; P^{38}-stathmin, serine-38 phosphorylated stathmin 1.

STMN1 induction might function to promote hepatocyte proliferation after liver injury. To examine the proliferative effects of STMN1 in the liver, mice were infected with AdStath or AdGFP as a control for the nonspecific effects of adenoviral infection. Successful viral infection and STMN1 expression were confirmed by qRT-PCR, which demonstrated significantly increased hepatic levels of *Stmn1* mRNA within 1 day of infection that peaked at 3 days and remained elevated for 11 days (Fig. 2A). Increased hepatic STMN1 protein content was also detected by immunoblotting within 1 day of infection with AdStath (Fig. 2B). STMN1 protein expression persisted for the 11 days of the study period, but levels began to decrease by day 9 (Fig. 2C). Immunofluorescence microscopy confirmed an absence of STMN1 staining in AdGFP-infected livers and the presence of hepatocyte STMN1 expression with AdStath infection (Fig. 2D). Infection with AdStath, therefore, rapidly and effectively increased hepatocyte STMN1 levels *in vivo*.

At 9 days after adenovirus infection, livers of mice infected with AdStath had a normal appearing surface but were greater in size than livers from AdGFP-infected mice (Fig. 3A). After infection, both the gross liver weight (Fig. 3B) and the ratio of liver to body weight (Fig. 3C) increased significantly in STMN1-expressing livers. An increase was detected beginning at 1.5 days and peaked at 9 days after adenovirus infection. The increase in mass persisted for 15 days, but liver size normalized by day 30 (Fig. 3D). Infection with AdGFP had no effect on liver weight or the liver to body weight ratio at any time point (Fig. 3B-D). Hepatocyte STMN1 expression induced a significant spontaneous increase in liver mass.

During adenovirus amplification, it is possible for wild-type E1A-positive adenovirus to be generated by homologous recombination between the virus vector backbone and the *E1a* gene in host HEK293 cells. Wild-type adenovirus has been reported to induce proliferation in primary islet cells.[21] We therefore

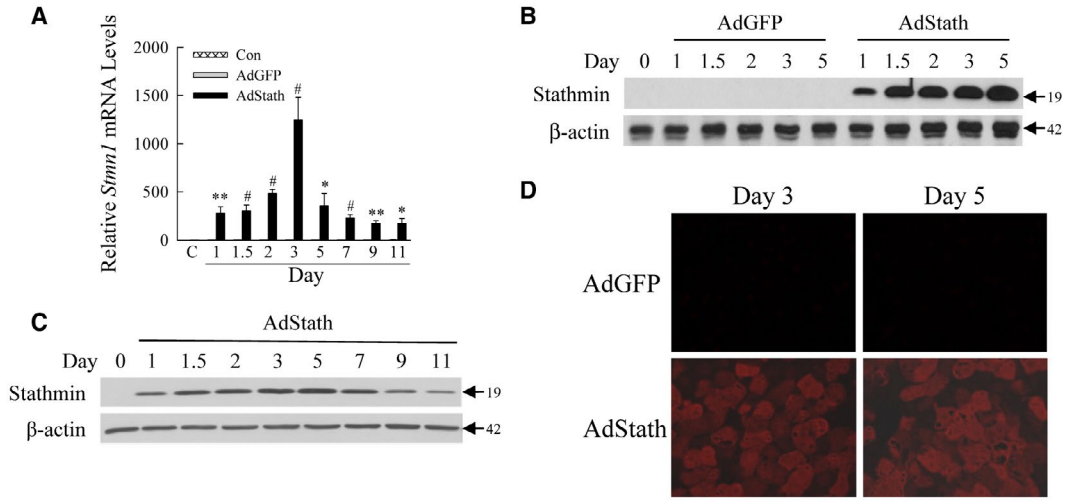

FIG. 2. AdStath infection increases hepatocyte STMN1 expression in mice. (A) Levels of *Stmn1* mRNA relative to uninjected control liver in mouse livers at the indicated days after AdGFP or AdStath infection (**P* < 0.05, ***P* < 0.01, #*P* < 0.001, compared to AdGFP-infected mice at the same time point; n = 3-6). (B,C) Immunoblots of total hepatic protein from uninfected mice (day 0) and mice infected with AdGFP or AdStath for the days indicated and probed for STMN1 and β-actin. (D) Immunofluorescence images of mouse livers infected with AdGFP or AdStath for 3 and 5 days stained for STMN1 (original magnification ×400). Protein bands and molecular weights in kilodaltons are indicated by arrows in (B,C). Abbreviation: C/Con, control.

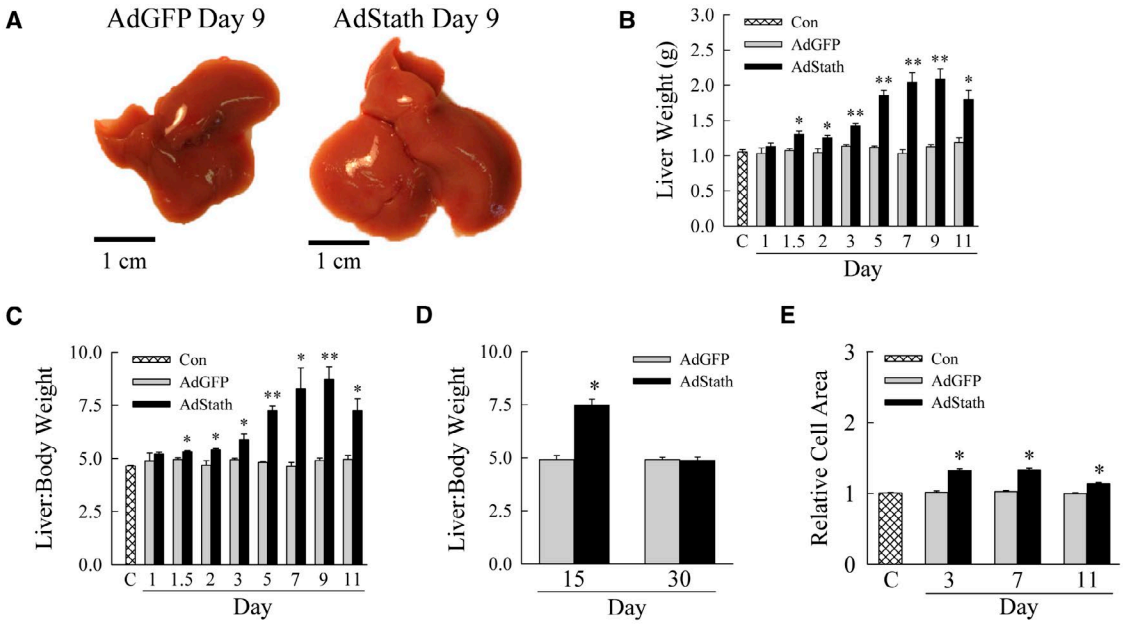

FIG. 3. Adenoviral STMN1 expression increases liver mass. (A) Gross images of representative mouse livers 9 days after injection with the adenovirus AdGFP or AdStath. Scale bar, 1 cm. (B) Liver weights and (C) liver to body weight ratios in uninjected control mice and mice at the indicated days after injection with AdGFP or AdStath (**P* < 0.01, ***P* < 0.001, compared to AdGFP-infected mice at the same time point; n = 4-8). (D) Liver to body weight ratios at later times (**P* < 0.01 compared to AdGFP-infected mice at the same time point; n = 4). (E) Relative hepatocyte cell size in adenovirus-infected cells (**P* < 0.000001 compared to AdGFP-infected mice at the same time point; n = 340-890). Abbreviation: C/Con, control.

examined whether wild-type adenovirus contamination was responsible for the proliferative effects of AdStath. The AdStath isolate failed to form plaques in A549 cells, and *E1a* mRNA was not detected in AdStath-infected livers. The increase in liver mass from AdStath was not secondary to wild-type adenovirus contamination.

STMN1 EXPRESSION *IN VIVO* DRIVES HEPATOCYTES INTO THE CELL CYCLE

Increased liver mass could result from hepatocyte hypertrophy and/or hyperplasia. To assess for hypertrophy, hepatocyte size was quantitated by fluorescence microscopy of livers stained for E-cadherin, an adhesion molecule that outlines cell borders.[22] AdStath-infected cells had a 33% increase in cell size over days 3 and 7 that decreased by day 11, indicating that cell hypertrophy had occurred (Fig. 3E).

To examine for hyperplasia from hepatocyte proliferation, the number of cells undergoing DNA synthesis was determined by BrdU staining. AdGFP-infected mouse livers had low numbers of BrdU-positive cells that were unchanged from levels in uninfected mice (Fig. 4A,B). In contrast, AdStath-infected livers had a slight but significant increase in BrdU staining within 1 day after infection (Fig. 4A,B). The number of BrdU-positive cells increased markedly between 1.5 and 3 days after AdStath infection and remained modestly but significantly elevated through day 11 (Fig. 4B). An effect of adenoviral STMN1 expression on cell proliferation was confirmed by histologic determination of the number of mitotic hepatocytes. Mitoses were rare in AdGFP-infected livers, but the number of mitotic hepatocytes increased significantly in AdStath-infected livers within 3 days after infection and remained elevated through day 11 (Fig. 4C,D).

To demonstrate a direct effect of STMN1 on the hepatocyte cell cycle, nuclear levels of cell-cycle genes were determined. In agreement with the BrdU staining, nuclear cyclin protein levels were increased in AdStath-infected livers at 1.5 days. Although nuclear levels of cyclin D1 and D2 were unchanged, D3 levels increased within 1.5 days of infection (Fig. 4E). Levels of cyclins A and B1 increased in concert with D3 (Fig. 4E). Nuclear levels of CDK2 and CDK4 similarly increased in AdStath-infected livers as did E2F transcription factor 2 (Fig. 4E). STMN1 expression was sufficient by itself to drive cyclin activation and entry of hepatocytes into the cell cycle in the absence of any prior reduction in liver mass or exogenous growth factor administration.

APOPTOSIS FOLLOWS THE INDUCTION OF HEPATOCYTE PROLIFERATION BY STMN1

Hepatocyte proliferation was followed by a cell death response in the livers of AdStath-infected mice. Apoptotic cells were seen on histology in AdStath-infected but not AdGFP-infected livers starting 3 days after infection and continuing for the 11 days of the study (Fig. 5A,B). The presence of apoptosis was further confirmed by findings of the active cleaved forms of effector caspases 3 and 7 in AdStath-infected livers (Fig. 5C). At 5 days following infection, liver injury occurred as indicated by a dramatic increase in serum ALT levels in AdStath-infected mice whereas ALTs were normal in AdGFP-infected mice (Fig. 5D). The onset of liver injury was associated with an inflammatory response. Occasional foci of inflammation were detected on histology in livers infected with either adenovirus, indicating a low level of nonspecific inflammation secondary to the adenoviral infection itself. However, there was a slight but significant increase at day 3 and a marked increase at day 5 in inflammation in AdStath-infected livers that persisted through day 11 (Fig. 5A,E). The STMN1 induction of hepatocyte proliferation and increase in liver mass were therefore followed by an apoptotic hepatocyte death response and liver injury and inflammation.

Discussion

A loss of adequate functioning liver mass leads to hepatic failure that underlies much of the morbidity and mortality from human liver disease. Studies have attempted to identify initiators of hepatocyte proliferation with the hope of developing therapies that stimulate liver regeneration in order to prevent liver failure. However, no single signal has been identified that initiates liver growth.[23] The present study identifies the ability of a single hepatocyte gene, *Stmn1,* to trigger the entry of quiescent hepatocytes into the cell cycle and cause a significant increase in liver mass. Hepatocyte hypertrophy also occurred but was

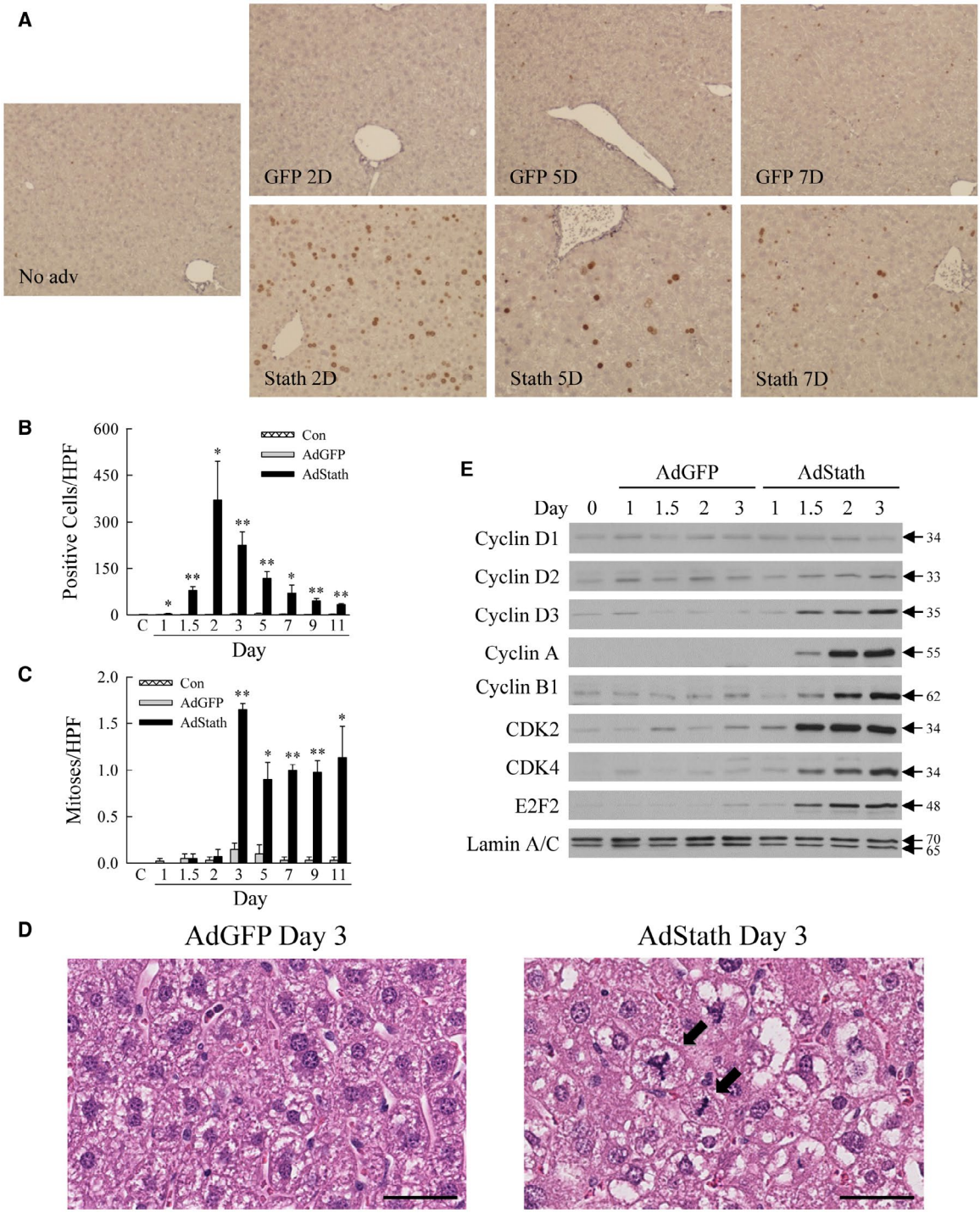

FIG. 4. STMN1 expression increases hepatocyte proliferation. (A) Images of BrdU-stained cells from AdGFP-infected (shown as GFP) and AdStath-infected (shown as Stath) mice at the indicated days after infection (magnification ×200). (B) Numbers of BrdU-positive cells per HPF (200×) in uninjected control mice and mice at the indicated days after AdGFP or AdStath injection (**P* < 0.04, ***P* < 0.001, compared to AdGFP-infected mice at the same time point; n = 4-8). (C) Numbers of mitoses per HPF (400×) by histology in the same mice (**P* < 0.03, ***P* < 0.002, compared to AdGFP-infected mice at the same time point; n = 3-6). (D) Representative hematoxylin and eosin images of adenovirus-infected livers (arrows indicate mitotic hepatocytes; scale bars, 50 μm). (E) Immunoblots of liver nuclear protein from an uninjected liver (day 0) and adenovirus-injected livers at the indicated days. Lamin A/C is a loading control. Arrows indicate the protein molecular weights in kilodaltons. Abbreviations: adv, adenovirus; C/Con, control; D, days; HPF, high-power field.

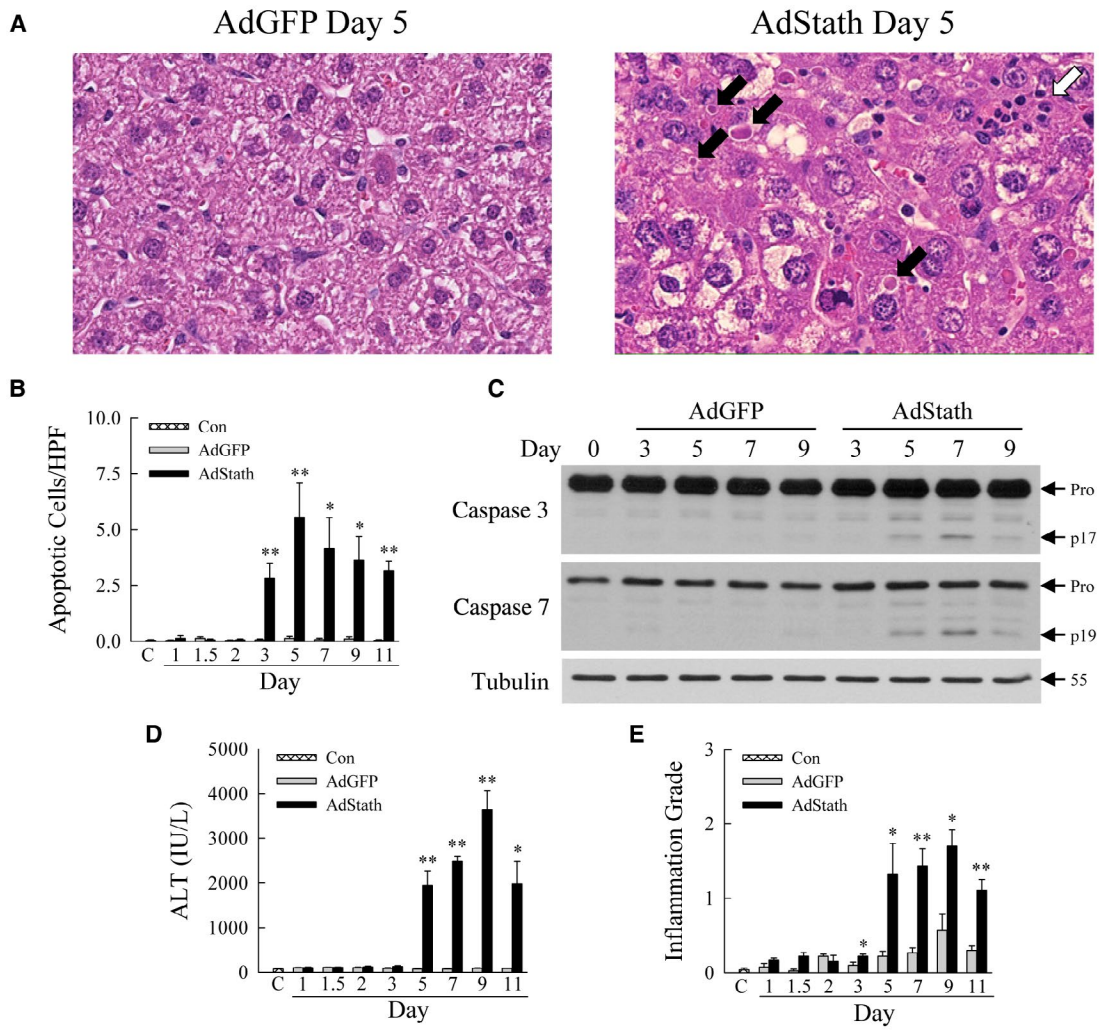

FIG. 5. Hepatocyte apoptosis and liver injury occur after hepatocyte proliferation. (A) Representative hematoxylin and eosin images of adenovirus-infected livers at day 5 400×; (black arrows indicate apoptotic hepatocytes, white arrow an area of inflammation). (B) Numbers of apoptotic cells per HPF (400×) by histology in uninjected control mice and AdGFP- or AdStath-injected mice at the indicated days (*P < 0.05, **P < 0.01, compared to AdGFP-infected mice at the same time; n = 3-4). (C) Immunoblots of total hepatic protein from uninfected mice (day 0) and mice infected with AdGFP or AdStath for the days indicated probed for caspase 3, caspase 7, and tubulin. Arrows indicate the procaspase and cleaved active forms. (D) Serum ALT levels in the same mice (*P < 0.01, **P < 0.001, compared to AdGFP-infected mice at the same time; n = 4-7). (E) Inflammation grade in the livers (*P < 0.05, **P < 0.01, compared to AdGFP-infected mice at the same time; n = 3-4). Abbreviations: C/Con, control; Casp, caspase; HFP, high-power field; IU, international unit; p17, cleaved form of caspase 3; p19, cleaved form of caspase 7; Pro, procaspase.

relatively modest compared to the marked increase in proliferation. Hepatocyte hypertrophy typically accompanies hepatocyte proliferation in other models of liver growth, such as partial hepatectomy.[24] Over time, the liver returned to a normal size due to the loss of adenoviral STMN1 expression and an apoptotic response. That the proliferative effect occurred in livers of normal size indicates that *Stmn1* expression by itself in the absence of the stimulus of a loss of functional liver tissue can increase liver mass. This capability separates STMN1 from other identified hepatocyte factors that promote hepatocyte proliferation but are incapable of initiating this process alone.

STMN1 has been implicated in cell proliferation through its potential to control entry into mitosis and therefore subsequent cell-cycle progression by regulating microtubules that form the mitotic spindle required for chromosome segregation.[25] The effects

of STMN1 on microtubules are complex and affected differentially by changes in levels of unphosphorylated versus phosphorylated protein. Dephosphorylated STMN1 destabilizes microtubules by binding to tubulin dimers to prevent their incorporation into microtubules and to microtubules themselves to stimulate plus-end catastrophes. Phosphorylation blocks STMN1 association with tubulin, thereby promoting microtubule stabilization and polymerization. In murine and human liver injury, both total and phosphorylated STMN1 levels are increased, making the net effect on microtubule formation unclear. In transformed cells, either increased or decreased levels of STMN1 can cause G_2/mitotic phase (M) arrest and reduce cell proliferation as the result of variable levels of phosphorylation. Overexpression of STMN1 in K562 leukemic cells led to G_2/M arrest and growth suppression,[26] whereas a knockdown of stathmin in the same K562 cells and in esophageal carcinoma cells also caused G_2/M cell-cycle arrest.[27,28] Thus, the functional effect of STMN1 expression on cell-cycle progression is controversial based on findings from transformed cells in culture. The present study is the first to examine STMN1 effects in nontransformed cells *in vivo*. Our findings demonstrate that STMN1 expression in normal liver dramatically stimulates hepatocyte proliferation, as shown by increased BrdU incorporation, numbers of mitotic cells, and liver mass.

The proliferative effect of STMN1 occurred through a mechanism other than the facilitation of cell entry into mitosis as normal hepatocytes are quiescent and require a signal to exit G_0 and enter G_1. In addition, the up-regulation of cyclins and cyclin kinases upstream of M phase occurred from *Stmn1* expression, indicating an effect prior to mitosis. A few studies of tumor suppressor genes have suggested that STMN1 may regulate cell proliferation through cell-cycle effects distinct from those on mitosis.[6] The tumor suppressor leucine-rich repeat 4 acts, in part, by down-regulating STMN1 expression, which functions to increase cyclin B and D1 expression.[29] The CDK inhibitor cyclin-dependent kinase inhibitor 1B ($p27^{kip1}$) negatively regulates the cell cycle by binding stathmin to block the G_1 to synthesis (S) phase transition.[30] The exact mechanism by which STMN1 promotes hepatocyte cell-cycle progression is unclear but occurs early in the cell cycle as nuclear cyclin D levels are increased in the stathmin-expressing livers.

The *Stmn1*-induced changes in cyclin expression differ somewhat from the pattern seen with the proliferative stimulus of liver mass reduction by partial hepatectomy. Nuclear levels of the cell-cycle regulators cyclins A1 and B increased in concert with the *Stmn1*-induced hepatocyte proliferative response similar to findings for these proteins during partial hepatectomy.[31-34] In contrast, the findings for the D-type cyclins in *Stmn1*-expressing mouse liver are surprising in that the increase occurred in cyclin D3 and not D1 at the times that were examined. All three D cyclins are similar in structure and function but have cell-type differences in expression.[35] The three have distinct biological effects, but all promote hepatocyte proliferation.[36] Cyclin D1 is considered an important factor in hepatocyte G_1 progression and is up-regulated during this phase of the cell cycle after partial hepatectomy, whereas D3 is elevated to a lesser extent.[34,37] Interestingly, STMN1-mediated hepatic proliferation occurred without apparent up-regulation of cyclin D1 but with increased nuclear cyclin D3, suggesting that D3 function may be sufficient for hepatocyte proliferation. Identical to findings in partial hepatectomy, no increase occurred in cyclin D2.[34] Studies of adenoviral expression of each individual cyclin D family member have demonstrated that all three stimulate hepatocyte proliferation, but cyclin D3 may be less effective in inducing DNA synthesis than the other isoforms.[36] This pattern of cyclin D regulation suggests the possibility of a distinct pathway of hepatocyte proliferation in response to STMN1 that differs from that following surgical reduction. Effects unique to *Stmn1* expression may explain the ability of this gene to drive hepatocytes into the cell cycle in the absence of any other cofactors.

After the initial increase in hepatocyte proliferation, STMN1 expression was associated with hepatocyte apoptosis. This finding was surprising in light of our previous *in vitro* data in which STMN1 expression prevented hepatocyte apoptotic and necrotic death from menadione-induced oxidant stress, indicating a protective survival role for STMN1 during hepatocyte injury.[14] Other studies support this prosurvival function because the silencing of STMN1 in a number of transformed cell lines, including breast, nasopharyngeal, and pancreatic cells, induced apoptosis in these cells.[38-40] The onset of apoptosis in STMN1-expressing livers occurred well after the initiation of cell proliferation and increase in liver mass. This temporal sequence suggests that apoptosis was not triggered as the direct

result of *Stmn1* expression but rather in response to the increase in liver mass. Undefined mechanisms that prevent hepatic overgrowth were likely activated in response to the increase in mass and triggered hepatocyte apoptosis to normalize liver size. Apoptosis in *Stmn1*-expressing mice was followed by liver injury and inflammation. Although apoptosis was originally considered a noninflammatory form of cell death because of a presumed failure of apoptotic hepatocytes to release proinflammatory factors, the ability of apoptosis to trigger inflammation in the liver has been well established.[41] A similar induction of apoptosis and increased ALTs has been described after partial hepatectomy.[42,43] The phenotype of STMN1-expressing mice is therefore consistent with the concept of the hepatostat or the necessity of the liver to maintain a liver to body ratio that is maximal for normal homeostasis.[44] STMN1-induced liver overgrowth may provide a unique model to study factors that halt liver growth and maintain the hepatostat.

Stmn1 expression in the absence of any prior reduction in liver size or exogenous growth factor administration induces a hepatocyte proliferative response that significantly increases liver mass. STMN1 could therefore be a unique target to stimulate liver regeneration in hepatic failure. This strategy may not be effective in some instances of liver failure as increased endogenous STMN1 expression was detected in human livers undergoing acute fulminant hepatic failure. Alternatively, STMN1 expression could be employed in hepatocyte transplants to increase the efficiency of liver replacement. STMN1 could have the dual beneficial effects of promoting transplanted cell survival and proliferation.

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Author names in bold designate shared co-first authorship.